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Rapid report

Identification of a novel mutation in exon 13 of the LDL receptor gene causing familial hypercholesterolemia in two spanish families

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Abstract

DNA from 30 unrelated Spanish patients with familial hypercholesterolemia (FH) was studied by single-strand conformation polymorphisms (SSCP)/heteroduplex analysis for mutation detection in exon 13 of low density lipoprotein (LDL) receptor gene. Two patients were found to have an abnormal pattern by heteroduplex analysis, and direct sequencing revealed a C to G substitution at nucleotide position 1965, that results in a Phe to Leu change in codon 634, F634L. We have developed a PCR based assay to detect this mutation in family members. We found three additional F634L mutation carriers, and all of them had high cholesterol levels. Haplotype analysis revealed that all F634L mutation carriers had the same allele determined by *Taq*I −, *Stu*I +, *Ava*II +, *Nco*I −, suggesting the presence of a common ancestor. We report a novel mutation located in exon 13 of the LDL receptor gene that causes FH. We also demonstrate the importance of combining SSCP and heteroduplex analysis to improve mutation detection.

Keywords: Familial hypercholesterolemia; LDLR mutation; SSCP analysis; (Spain)

Familial hypercholesterolemia (FH) is inherited in an autosomal dominant manner and is one of the most common genetic diseases. It is caused by heterogeneous mutations in the low density lipoprotein (LDL) receptor gene [1]. The majority of mutations causing FH are point mutations or small deletions/duplications located along the 45 kilobases (kb) of the LDL receptor gene [2]. Analysis of single-strand conformation polymorphisms (SSCP) has proved to be a powerful tool to detect point mutations in the LDL receptor gene [3,4].

As part of a project aimed at characterizing mutations in the LDL receptor gene underlying FH in Spain, we have applied single-strand conformation polymorphisms (SSCP) and heteroduplex analysis [5] to screen 30 unrelated Spanish FH patients for point mutations in exon 13 of the LDL receptor gene. We report here a novel mutation in exon 13 causing FH. This point mutation results in substitution of phenylalanine for leucine at amino acid 634 of the LDL receptor.

Using a previously described method [6], we isolated genomic DNA from peripheral blood from the FH patients sample. A fragment of the LDL receptor gene comprising exon 13 and its flanking regions (329 bp) was amplified using the polymerase chain reaction (PCR). We used the oligonucleotides 5′-GTCATCTTCCTTGCTGCCTG-3′ and 5′-TTCCAC-AAGGAGGTTTCAAGGTTGGGGGGG-3′, and we subjected the genomic DNA to 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 74°C for 3 min, followed by a final extension of 10 min at 74°C. For SSCP and heteroduplex analysis, PCR products were radiolabelled with ^{32}P-dCTP, added to a formamide loading buffer, denatured at 94°C for 4 min, and electrophoresed at 6 W for 16 h, at room temperature, on a HydroLink-MDE gel (AT Biochem, Malvern, PA) with or without 5% glycerol, in a 0.6 × TBE buffer. The electrophoresis was stopped before the double strand DNA fragments reached the bottom of the gel, in order to be able to detect heteroduplex formation. The gels

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were dried and autoradiographed for 3–4 days. Direct sequencing was performed in an ABI DNA Sequencer (Model 373A) with a dye terminator kit, as previously described [7]. Nucleotide positions were numbered as suggested by Yamamoto et al. [8].

To detect the presence of the C to G substitution in position 1965, a PCR based assay was carried out amplifying a 255 bp fragment with primers 5′-AGTGCCAACCGCCTCACAGG-3′ and 5′-CCTCTCACACCAGTTCACTC-3′, and denaturing for 1 min at 96°C, annealing for 2 min at 62°C, and extending for 1 min 30 s at 74°C. This heating cycle was repeated 30 times followed by a final extension of 10 min at 74°C. An aliquot of 15 μl of this PCR product was digested with 10 U of *Mbo*II at 37°C for 3 h. Following electrophoresis on 8% polyacrylamide gel, the digestion products were visualized with ethidium bromide. The haplotypes of FH family members were determined using 4 polymorphisms in the LDL receptor gene. The variable restriction enzyme sites used were: TaqI in intron 4 [9], StuI in exon 8 [10], AvaII in exon 13 [11] and NcoI in exon 18 [12]. These polymorphisms were detected by PCR amplification and restriction enzyme digestion. Alleles were designated as '+' to indicate presence or '−' to indicate absence of the restriction site.

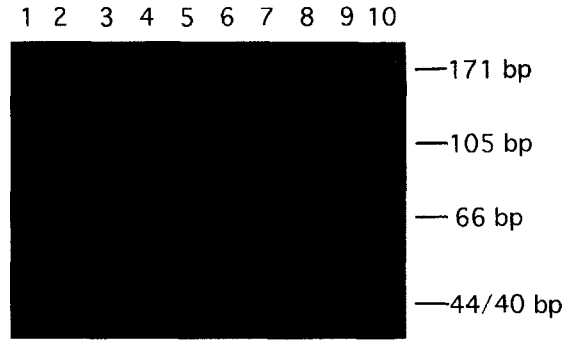

Fig. 2. Analysis of F634L mutation by PCR. Ethidium bromide stained 8% polyacrylamide gel after electrophoresis of *Mbo*II digested PCR products. F634L mutation destroys a *Mbo*II cleavage site that is present in the normal allele. After cleavage with *Mbo*II, either a 105, 66, 44 and 40 bp fragments (wild type) or 171, 105, 66, 44 and 40 bp fragments (heterozygous mutant type) are produced. Lanes 1, 4, 6, 7, 10: Heterozygous F634L carriers; lanes 2, 3, 5, 8, 9: Normal relatives.

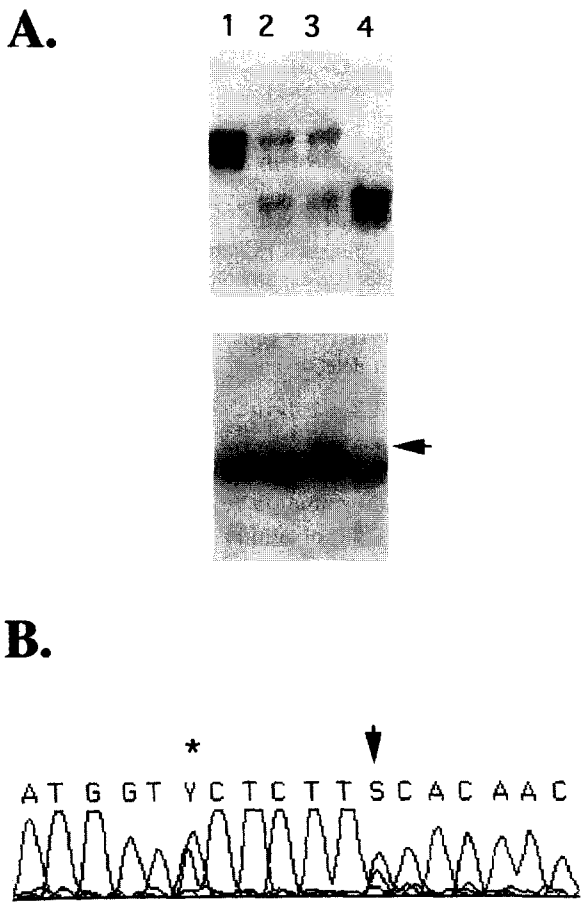

Fig. 1. SSCP/heteroduplex and sequence analysis of exon 13 of the LDL receptor gene. A. SSCP/heteroduplex patterns were observed without glycerol in the gel. Lanes 1 and 4: Control individuals homozygous for *Ava*II polymorphism, harboring a T or a C in position 1959, respectively. Lane 2: Control individual heterozygous for *Ava*II polymorphism, harboring T/C in position 1959. Lane 3: FH heterozygous person harboring T/C in position 1959 and C/G in position 1965. Arrow indicates an extra band in heteroduplex analysis. B. Sequence of nucleotides 1954–1971 corresponding to lane 3 subject in panel A. Asterisk indicates *Ava*II polymorphism. Arrow indicates F634L mutation.

SSCP-heteroduplex analysis and sequencing results are shown in Fig. 1. In the upper part of the gel (Fig. 1A), corresponding to SSCP analysis, no abnormal pattern due to a mutation can be distinguished. However, in the lower part or the gel, corresponding to heteroduplex formation, an extra band due to a nucleotide change is detected in two patients, PB I.1 and RM I.2. Direct sequencing of the exon 13 of the LDL receptor gene revealed a C to G transversion in position 1965 that changes codon 634 from phenylalanine to leucine, F634L (Fig. 1B). A PCR based assay to detect the mutation F634L is shown in Fig. 2. The wild type exhibited 105, 66, 44 and 40 bp fragments after the 255 bp PCR product was digested with *Mbo*II restriction enzyme. The F634L mutation destroys a *Mbo*II restriction site, and 171, 44 and 40 bp fragments are produced in the mutant allele. We studied all available family members of the two unrelated probands carrying the mutation. In both families PB and RM, 2 and 1 additional heterozygous FH patients with the F634L mutation, respectively, were identified. Pedigrees of these two families are shown in Fig. 3.

The lipid levels of family members studied are shown in Table 1. Compared with the general Spanish population [13,14], all the F634L carriers had total and LDL cholesterol levels above the 95th percentile. In both FH families, lipid levels were higher in mutation carriers than in relatives without the F634L mutation. There were no significant differences in HDL cholesterol and triglycerides between mutation carriers and non-mutation carriers.

The LDL receptor genotypes of the family members were determined at four polymorphic sites within the LDL

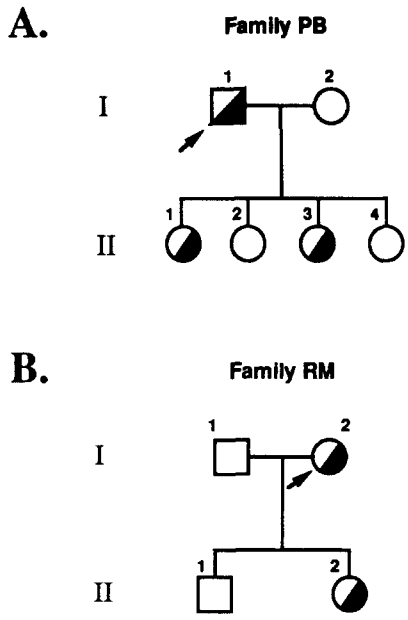

Fig. 3. Pedigrees of families PB and RM with the F634L mutation. The half-filled symbols represent the F634L mutation carriers. Arrow indicates probands.

receptor gene. The haplotypes were deduced from the genotypes of relatives assuming there were no recombination events within the family. The haplotype of the allele carrying the F634L mutation was *Taq*I −, *Stu*I +, *Ava*II +, *Nco*I −.

There are more than 150 different mutations in the LDL receptor gene causing FH. Many of these mutations disrupt receptor function in different ways that reveal much about the relationship between receptor structure and function [2]. In this work, we describe a novel mutation located in exon 13 of the LDL receptor gene. Exon 13 codes for a portion of the human epidermal growth factor (EGF) precursor homology domain. This domain includes three growth factor repeats, which are 40 amino acid cysteine-rich sequences. The first two growth factor repeats (A and B) are separated from the third growth factor repeat (C) by a 280 amino acid sequence that contains five copies of a conserved motif, Tyr-Trp-Thr-Asp, repeated once each 40 to 60 amino acids [1]. The EGF precursor homology domain is required for the acid-dependent dissociation of lipoproteins from the receptor in the endosome during receptor recycling. It also serves to position the ligand binding domain so that it can bind LDL on the cell surface [15]. This domain is very highly conserved, and it has a compact structure that is easily disrupted even if only one single amino acid is changed [16]. Exon 13 codes for 47 amino acid sequence at the end of the spacer region, before the third growth factor repeat (C). The missense mutation F634L identified in codon 634 probably disrupts the protein folding in a such way that transport of LDL receptor from endoplasmic reticulum to Golgi complex is blocked, like other mutations described in the EGF domain [2], and it is expected to cause class 2 alleles.

The F634L mutation segregates with high lipid levels in the two families studied, and all the mutation carriers had lipid levels above the 95th percentile compared with the general Spanish population [13,14]. Furthermore, one of the probands carrying the mutation, RM I.2, had the highest total and LDL cholesterol levels in the sample of 30 heterozygous FH subjects studied (14.8 and 13.1 mmol/l, respectively). Therefore, the F634L mutation seems to be pathogenic and the cause of FH, as no other mutation in the rest of the gene could be detected in these two subjects by SSCP/heteroduplex and Southern blot analyses (data not shown). The Phe to Leu change does not involve any amino acid of the conserved motif Tyr-Trp-Thr-Asp, but the Phe in position 634 could be important for the correct folding of the protein.

Only one mutation in exon 13 of the LDL receptor gene has been reported previously in a Chinese patient using SSCP [17]. We could not detect the F634L mutation by SSCP analysis only. However, using SSCP together with heteroduplex analysis as described above, we were able to detect an extra band corresponding to a nucleotide substitution. It is possible that the F634L mutation is refractory to detection by SSCP analysis due to the fact that it is only 6 nucleotides downstream of the *Ava*II polymorphism [11], and both unrelated subjects carrying this mutation were heterozygous for this polymorphic site. In contrast, the A606T mutation identified in the Chinese subject is 80 nucleotides upstream of the *Ava*II polymorphism [17]. The genotype of this patient at that polymorphic site is not available, and thus, if the subject was homozygous for the *Ava*II site, the mutation would be easily detected by SSCP. It seems possible that other mutations in exon 13 lying in proximity to the *Ava*II polymorphism in heterozygous subjects could also be refractory to detection by SSCP analysis.

Table 1
Lipid values for the two families carrying the F634L mutation

Subject	Sex	Age	TC	LDL-C	HDL-C	TG
Family PB						
I.1 *	m	47	10.2	8.6	1.2	0.8
I.2	f	45	5.6	3.8	1.4	0.7
II.1 *	f	21	8.9	7.1	1.4	0.8
II.2	f	20	4.8	2.8	1.7	0.7
II.3 *	f	15	7.1	5.3	1.5	0.6
II.4	f	13	4.7	2.9	1.5	0.6
Family RM						
I.1	m	61	5.6	3.6	0.9	2.2
I.2 *	f	65	14.8	13.1	1.0	1.4
II.1	m	33	5.4	3.4	1.4	1.2
II.2 *	f	27	8.8	6.5	1.7	1.1

TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglycerides, all measured in mmol/l. Asterisk indicates subjects carrying F634L mutation.

In conclusion, a novel pathogenic mutation in exon 13 of the LDL receptor gene is described. Further characterization of this F634L mutation will help us to understand better the functional properties of this portion of the EGF precursor homology domain.

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